Departement für Nutztiere

der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. med. vet. Dr. med. vet. h.c. Ueli Braun

Veterinärmedizinisches Labor

Leiterin: Prof. Dr. med. vet. Regina Hofmann-Lehmann

Arbeit unter wissenschaftlicher Betreuung von PD Dr. med. vet. Barbara Willi Andris

The genetic diversity and phenotypic associations of feline caliciviruses from cats in Switzerland

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vorgelegt von

Andrea Monika Spiri

Tierärztin

von Amlikon-Bissegg, TG

genehmigt auf Antrag von

Prof. Dr. med. vet. Regina Hofmann-Lehmann, Referentin

Prof. Dr. med. vet. Felicitas S. Boretti, Korreferentin

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Abstract

Feline calicivirus (FCV) is one of the most common viral pathogens in domestic cats worldwide. The variable regions of the capsid (VP1) gene of FCV have one of the highest recorded rates of molecular evolution and therefore favouring rapid viral evolution. In this study we undertook, for the first time, phylogenetic analyses using the near-complete capsid gene. Sequences from 66 Swiss FCV isolates were used to investigate the correlation between viral phylogeny and several traits (i.e. geographic origin, signalment, husbandry and FCV vaccination). Codon-based nucleotide alignment showed that individual nucleotides and their corresponding amino acid sites were either invariant or highly variable. Using a threshold of 20% genetic distance in the variable region E, FCV isolates grouped in 52 strains, 10 of which comprised 2 to 3 isolates. Two well-supported lineages (named 1 and 2) were found and contained 9 and 24 isolates, respectively. A strong association between cat pedigree status and FCV genetic diversity was found. Most of the FCV isolates from pedigree cats belonged to lineage 2. Possible explanations for the existence of genetically related FCV isolates in pedigree cats include a shared transmission route or genetic characteristics of pedigree cats that might increase susceptibility to certain FCV strains. The phylogenetic resolution in the present study surpassed that of previous ones due to the inclusion of longer and more conserved sequences.
Key words: Feline calicivirus, pedigree cat, phylogenetic analyses, trait-correlation, capsid (VP1) gene

Zusammenfassung

Feline Caliciviren (FCV) gehören zu den häufigsten viralen Pathogenen bei Katzen weltweit. Die variable Region des Kapsidgenes (VP1) von FCV weist eine der höchsten Evolutionsraten für Viren auf, was zu einer schnellen genetischen Weiterentwicklung von FCV führt. In dieser Studie wurden zum ersten Mal phylogenetische Analysen basierend auf dem fast kompletten Kapsidgen durchgeführt. Die Sequenzen von 66 Schweizer FCV Isolaten wurden erhoben und die Phylogenie mit diversen Merkmalen der Katzen (geografische Herkunft, Signalment, Haltungsformen, FCV Impfung) korreliert. Das Codon-basierte Nukleotid Alignment zeigte, dass die einzelnen Nukleotide und die korrespondierenden Aminosäuren entweder identisch oder sehr variabel waren. Basierend auf einer genetischen Distanz über 20% in der variablen Region E des Kapsidgenes wurden die Isolate in 52 Stämme eingeteilt, wobei zehn Stämme zwei bis drei Isolate enthalten. Der phylogenetische Baum zeigte zwei gut unterstützte Abstammungslinien (1 und 2), die 9 respektive 24 Isolate enthalten. Es wurde eine starke Assoziation zwischen Reinrassigkeit und FCV Phylogenie gefunden. Fast alle Isolate von Rassekatzen gruppierten in die Abstammungslinie 2. Ein gemeinsamer Übertragungsweg oder die genetische Konstitution sind mögliche Erklärungen für das Vorkommen von genetisch verwandten FCV Isolaten in Rassekatzen. Die phylogenetische Auflösung war höher als in bisherigen Studien aufgrund der Analyse von längeren und konservierteren Sequenzen.

Schlüsselwörter: Felines Calicivirus, Rassekatzen, phylogenetische Analysen, Merkmal-Korrelation, Kapsid (VP1) Gen

The genetic diversity and phenotypic associations of feline caliciviruses from cats in Switzerland: Andrea M. Spiri, Julien Thézé, Marina L. Meli, Valentino Cattori, Alice Berger, Adolf Steinrigl, Oliver G. Pybus, Regina Hofmann-Lehmann, Barbara Willi; submitted to Journal of Virology, 2016

Andrea M. Spiri[a,b,*], Julien Thézé[c,*], Marina L. Meli[a,b], Valentino Cattori[a,b], Alice Berger[a,b], Adolf Steinrigl[d], Oliver G. Pybus[c], Regina Hofmann-Lehmann[a,b], Barbara Willi[a,e,#]

[a]Clinical Laboratory, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland, [b]Center for Clinical Studies, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland, [c]Department of Zoology, University of Oxford, South Parks Road, Oxford, OX1 3PS, UK, [d]Institute for Veterinary Disease Control Mödling, AGES – Austrian Agency for Health and Food Safety, Mödling, Austria and [e]Clinic for Small Animal Internal Medicine, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland

*these authors contributed equally

#corresponding author

Mailing address: Clinical Laboratory and Clinic for Small Animal Internal Medicine, Vetsuisse Faculty, University of Zurich, Winterthurerstr. 260, 8057 Zurich, Switzerland. Phone: +41 44 635 86 94. Fax: +41 44 635 89 06. E-mail: bwilli@vetclinics.uzh.ch

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Abstract

Feline calicivirus (FCV) is one of the most common viral pathogens in domestic cats worldwide. The variable regions of the capsid (VP1) gene of FCV have one of the highest recorded rates of molecular evolution, favouring rapid viral evolution and possibly supporting the emergence of novel, occasionally highly virulent strains. In this study, we undertook, for the first time, phylogenetic analyses using nearly complete capsid (VP1) gene sequences. Sequences from 66 Swiss FCV isolates were used to investigate the correlation between viral phylogeny and several traits, including geographic origin, signalment, husbandry, FCV vaccination, and co-infections. Codon-based nucleotide alignment showed that individual nucleotides and their corresponding amino acid sites were either invariant or highly variable. Using a threshold of 20% genetic distance in variable region E, FCV isolates were grouped into 52 strains, 10 of which comprised 2 to 3 isolates. Two well-supported lineages (named 1 and 2) were found and contained 9 and 24 isolates, respectively. A strong association between cat pedigree status and FCV genetic diversity was found; most of the pedigree cats were Maine Coons. Ancestral character state estimation showed that most (17/21) of the FCV isolates from pedigree cats belonged to lineage 2. No correlation between viral genetic distances and geographic distances was evident. Possible explanations for the existence of genetically related FCV isolates in pedigree cats include a shared transmission route (e.g., infection during cat shows) or genetic characteristics of pedigree cats that might increase their susceptibility to certain FCV strains.

Importance: Understanding the genetic diversity and phylogeny of FCV is a prerequisite to exploring the epidemiology and pathogenesis of this virus and to the

development of efficacious vaccine strategies. In this study, we undertook a nationwide molecular characterization of FCV using nearly complete capsid (VP1) gene sequences. We found significant associations between FCV phylogeny and host characteristics, specifically the pedigree status of the cats, and we identified two well-supported lineages in which the current FCV strain definition was confounded. The greater resolution of the FCV phylogeny in this study compared to previous studies can be attributed to our use of more conserved regions of the capsid (VP1) gene; nonetheless, our results were still hampered by sequence saturation. The study highlights the need for whole genome sequences for FCV phylogeny studies.

Key words: Feline calicivirus, pedigree cat, phylogenetic analyses, trait correlation, capsid (VP1) gene, hypervariable region E, strain

Introduction

Feline calicivirus (FCV) is a single-stranded, non-enveloped RNA virus belonging to the genus *Vesivirus* within the family *Caliciviridae* (1). Three other genera are assigned to the *Caliciviridae*, namely the genera *Sapovirus*, *Lagovirus* and *Norovirus.* Some of these genera contain highly virulent species, for example rabbit hemorrhagic disease virus or human norovirus, which is a major cause of gastroenteritis in adults and children (2). FCV is one of the most common viral pathogens of cats worldwide, and infections are associated with mucosal and cutaneous ulcerations, chronic oral inflammatory disease (gingivitis/stomatitis), pneumonia and a limping syndrome (3). Furthermore, outbreaks of highly virulent systemic FCV infections have been reported, first in the USA (4-6) and subsequently also in Europe (7-11). Affected cats develop a systemic inflammatory response syndrome and show high fever, subcutaneous oedema and skin ulcerations. Mortality rates of up to 60% have been reported for this syndrome (12). Despite these highly

virulent cases, asymptomatic FCV infections have been described, and up to 21% of healthy cats have been reported to test PCR-positive for FCV (13-15). Viral characteristics and host-specific factors are both thought to play a role in the development of FCV-associated disease. Several studies have tried to identify FCV nucleotide or amino acid sequence variations that are unique to different disease manifestations, but the genetic basis of FCV pathogenicity remains unresolved (11, 16-19).

The FCV genome comprises a 7.7 kb positive−sense RNA molecule that encodes three open reading frames (ORF). ORF 1 forms the viral replication complex, ORF 3 encodes the minor structural protein VP2 and ORF 2 encodes the major capsid protein VP1. ORF 2 can further be divided into six regions, A−F. Regions B, D and F are more conserved, whereas regions A, C and E are variable (1). Region E contains two hypervariable regions that are separated by a conserved domain (1). Variable regions C and E of the capsid gene exhibit one of the highest evolutionary rates reported for RNA viruses (20). These regions are of particular interest because they contain major neutralizing epitopes that define FCV antigenicity (21, 22). Furthermore, strain definition and phylogenetic analyses of FCV isolates have been based on variable regions C and E. Previous studies showed that epidemiologically related FCV isolates differ at less than 20% of the nucleotide sites in the C and E regions of the capsid (VP1) gene (23-26). Based on this 20% genetic distance threshold for FCV strain definition, remarkably high strain diversity was identified in the cat population in the UK and other European countries (27, 28). Phylogenetic analyses of variable regions C and E have been hampered by a lack of phylogenetic signal above the strain level caused by sequence saturation (27, 28). Previous studies were unable to draw reliable conclusions concerning phylogenetic

relationships above the strain level or to correlate FCV phylogeny with clinical syndromes or the geographic location of infected cats (27, 29, 30).

To overcome this limitation, in this study, we undertook a nationwide molecular characterization of FCV isolates using the nearly complete capsid (VP1) gene, which includes the more conserved regions B, D and F of the capsid (VP1) gene. We sequenced a total of 66 FCV isolates from cats from 17 cantons of Switzerland that showed different clinical manifestations of FCV infection. We investigated the association between FCV gene sequences and the location, signalment, husbandry, clinical signs and vaccination history of the cats.

Materials and methods

Study setup, sample collection and processing

The samples available for this study were collected during a nationwide FCV study that aimed to obtain a large collection of FCV isolates from symptomatic and asymptomatic cats in Switzerland (13). For this purpose, oropharyngeal cytobrushes, and nasal and conjunctival swabs were collected from 200 cats with suspected FCV infection and from 100 healthy cats in 24 veterinary practices in 17 cantons of Switzerland (13). Only one cat per owner was included. Cats that had been vaccinated within 21 days prior to collection were excluded. Samples from each cat were pooled, and total nucleic acid (TNA) was extracted as described (13). The data available for each cat contained information on the geographic origin, demographic data (age, sex, reproductive status, pedigree and breed), husbandry data (type of husbandry, such as private, cat breeder and others, group housing, outdoor access), vaccination history, medical treatments and clinical signs (13). Two suspected FCV cases (isolates 65 and 185) showed clinical signs resembling virulent systemic

disease; genetic data from these cats were obtained during a previous study (11). To compare our genetic data with that of commercial FCV vaccine strains, RNA was extracted from two commercial FCV vaccines (Feligen® CRP, Virbac, Glattbrugg, Switzerland; Nobivac® Tricat III, MSD Animal Health GmbH, Luzern, Switzerland). The vaccine material was resuspended in 280 µL of RNase/DNase-free molecular biology grade water (Axon Lab AG, Baden, Switzerland), and RNA was extracted using a QIAamp® Viral RNA kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's instructions.

Real-time qPCR and real-time RT-qPCR assays

Within the previous study (13), TNA was tested for the presence of FCV with two published real-time reverse-transcriptase (RT) quantitative (q) PCR assays (31, 32), named S1 and S2, that were recently optimized (13). All cats that tested positive in at least one of the two RT-qPCR runs were categorized as FCV-positive. Moreover, for each sample, the results for the detection of FHV-1, *Chlamydia felis*, *Mycoplasma felis* and *Bordetella bronchiseptica* by real-time qPCR assays were available (13). All real-time qPCR assays were run on an ABI 7500Fast Real-Time PCR system (Applied Biosystems, Rotkreuz, Switzerland). Negative and positive controls were included in each PCR run.

Synthesis of cDNA, conventional PCR amplification and sequencing

FCV-positive samples with high viral loads (cycle threshold values < 30.0; n = 75) in RT-qPCR S1 or S2 and RNA extracted from the two commercial FCV vaccines (Feligen® CRP, Virbac; Nobivac® Tricat III, MSD Animal Health GmbH) were used for sequencing (13). For this purpose, cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) under the following conditions:

10 min 25°C, 120 min 37°C, 10 min 70°C. The reaction mixture consisted of 2.5 µl 10× RT Buffer, 2.5 µl 10× RT random primers, 1.0 µL 25× dNTP Mix (100 mM), 1.25 µL MultiScribe® Reverse Transcriptase (50 U/µL), 0.3125 µL RNasin® Plus RNase Inhibitor (40 U/µL) (Promega AG, Dübendorf, Switzerland), and 10 µL template TNA, brought up to 25 µL with RNase/DNase-free molecular biology grade water (Axon Lab AG). FCV cDNA was amplified with Phusion™ Hot Start II High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, USA) using the published primers AoS (forward) and AoA (reverse) (33), which results in the amplification of 1945 nucleotides (nt) of the FCV capsid (VP1) gene (equivalent to nucleotide positions 5326 to 7270 of the FCV F9 reference strain; GenBank accession number M86379). The PCR reaction comprised 5 µL 5× Phusion HF Buffer, 0.5 µl dNTPs (10 mM), 0.625 µL primer AoS (20 µM) and 0.625 µL primer AoA (20 µM), 0.25 µL Phusion Hot Start II Polymerase (2 U/µL) and 2.5 µL of template cDNA brought up to 25 µL with RNase/DNase-free molecular biology grade water (Axon Lab AG). The PCR thermal cycling conditions were as follows: 30 sec at 98°C, 40 cycles of 10 sec at 98°C, 30 sec at 53°C and 90 sec at 72°C, followed by a final elongation for 10 min at 72°C.

In 17 samples, PCR amplification with the above-mentioned protocol failed. These samples were therefore amplified with the SuperScript® III One-Step RT-PCR System and the Platinum® *Taq* DNA polymerase (Invitrogen, Basel, Switzerland) using the primers AoS and AoA (33), which resulted in the successful amplification of 8/17 samples. For PCR amplification, the following reaction mixture was used: 12.5 µL 2× reaction Mix, 0.625 µL RNasin® Plus RNase Inhibitor (40 U/µL), 0.25 µL primer AoS (20 µM) and 0.25 µL primer AoA (20 µM), 1.0 µL SuperScript® III Platinum® *Taq* DNA polymerase and 5 µL of template TNA made up to 25 µL with RNase/DNase-free molecular biology grade water (Axon Lab AG). The RT step was

performed at 60°C for 30 min, followed by PCR amplification with an initial step at 94°C for 2 min, 40 cycles of 94°C for 15 sec, 53°C for 30 sec and 68°C for 150 sec, followed by 68°C for 5 min. The synthesis of cDNA and the conventional PCR were run on a Biometra TPersonal thermal cycler (BioLabo Scientific Instruments, Châtel-Saint-Denis, Switzerland). For each PCR run, the negative and positive controls were included to monitor for cross contamination and successful PCR amplification.

The PCR products were separated on a 1.5% agarose gel, and bands of the appropriate size (1945 bp) were excised and eluted with a QIAquick® Gel extraction kit (Qiagen) using 100 µL elution buffer. For sequencing, the amplification primers AoS and AoA (33) and newly designed internal primers were used (S_FCV_La.543f: 5'-GCT-TGG-TCT-GGM-TCT-ATT-GA-3'; S_FCV_Fl.1265r: 5'-GCC-AAC-CAT-CAG-GTA-TGC-CT-3'; FCVSeq_6145_6164f: 5'-CAY-YTD-ATG-TCT-GAY-ACT-GA-3'; FCVSeq_5749_5768f: 5'-GAR-CCH-ARY-KCH-CAA-ATG-TC-3'; FCVSeq_6705_6725r: 5'-GGR-ATK-GTD-GTR-TCD-GGC-CA-3'). Sequencing was performed at a commercial laboratory (Microsynth, Balgach, Switzerland) under standard conditions. Because direct sequencing of the capsid (VP1) gene was not successful in five samples (Nos. 52, 68, 100, 145 and 155), the PCR products of these samples were cloned using a Topo® TA Cloning® Kit with the pCR™ II-TOPO® vector and TOP10F' One Shot® *E.coli* bacteria (Invitrogen, Basel, Switzerland). Correct insertion of the PCR product was checked by EcoRI (Thermo Scientific) digestion analysis, and plasmid DNA was purified with a Qiaprep® Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. The inserted PCR products were sequenced at a commercial laboratory (Microsynth) using the sequencing primers described above.

Sequence alignment and phylogenetic analyses

All sequences were assembled, and a consensus sequence for each sample was obtained using Geneious 7.1.8 (34). The capsid (VP1) gene sequences of the vaccine strains FCV G1 and FCV 431 were provided by the manufacturer (Merial, Lyon, France). Reference FCV sequences from Europe, North America, Asia and Oceania were retrieved from GenBank. The European reference sequences included isolates from four cats from Switzerland or Liechtenstein that showed clinical signs compatible with virulent systemic disease (11). Codon-based multiple alignment of the capsid (VP1) nucleotide sequences was performed using MAFFT (35). Nucleotide sequence saturation tests were performed in Dambe (36), which implements Xia et al.'s test of nucleotide substitution saturation (37, 38). This test indicated that the 3rd codon positions in the nucleotide alignment were not saturated. The full nucleotide alignment was retained and used in phylogenetic analyses.

Bayesian and maximum likelihood (ML) phylogenetic trees were estimated from the nucleotide alignment with MrBayes (39) (with 10,000,000 generations and 25% burnin) and RAxML (40), respectively. In both cases, the GTR + G + I substitution model and parameters selected by jModelTest2 were used (41). Statistical support for nodes in the ML phylogeny was assessed using a bootstrap approach with 100 replicates. The trees were midpoint rooted. Given the known sampling dates of each isolate, the temporal signal of the phylogenies was assessed using TempEst (42). A regression of the sampling date against the root-to-tip genetic distances indicates that the data set contains insufficient temporal signal to justify the use of a phylogenetic molecular clock model (data not shown). The isolates were classified into FCV "strains" using the previously defined nucleotide distance threshold of 20% for variable region E of the capsid (VP1) gene (23, 25). Pairwise genetic distances

among isolate sequences were calculated using the function “dist.dna” (Jukes-Cantor model, JC69) implemented in the R package “ape” (43). Lineages were defined as well-supported clades containing at least two multiple-isolate strains.

Phylogeny-trait correlation and ancestral character estimations

Possible correlations between phylogenetic tree structure and trait values for each isolate (e.g., sampling location, vaccination status; see Tables 1-4) were assessed using the methods implemented in BaTS (44). Trait values were randomized 100 times to yield a null distribution for hypothesis testing. A correlation was considered unambiguously positive if both the association index (AI) and the parsimony score (PS) statistics rejected the null hypothesis with $p \leq 0.01$. Phylogenetic uncertainty was taken into account by using the set of tree topologies estimated by Bayesian phylogenetic inference (MrBayes). For those traits that were significantly clustered on the FCV phylogeny, parsimonious ancestral character state estimation was performed using the function “pace” implemented in the R package “phangorn” (45).

Comparison between genetic and geographical distances

Pairwise genetic distances between isolate sequences were calculated as described above. Geographical distances between each pair of isolates were calculated using the postcodes for each isolate and an online distance calculator (http://distancecalculator.globefeed.com). A quadratic assignment procedure correlation was used to test for a relationship between the genetic distance (%) and the geographical distance using the function “qaptest” implemented in the R package “sna” (46). This method computes standard measures of correlation between the genetic and geographic distance matrices and then computes an estimate of the significance of the correlation by permuting the elements of one of the matrices 5,000

times and counting the number of correlations between the observed and permuted matrices that are larger or smaller than the empirical estimate. The genetic and geographic distance matrices were plotted and visualized using the function “hexbin” implemented in the R package “hexbin” (47).

Statistical analysis

The exposure variables between pedigree and non-pedigree cats were compared using a Chi-squared ($p_{\chi 2}$) test or a Fisher’s exact test (p_F) for small numbers ($n < 5$) and Analyse-it® for Microsoft Excel 4.51 (Analyse-it Software Ltd., Leeds, United Kingdom, http://www.analyse-it.com). Proportions and 95% confidence intervals (CI) were calculated using GraphPad Prism® version 6 for Windows (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com). Variables such as the age of the cats were compared between the two groups using the Wilcoxon-Mann-Whitney test (p_{MWU}). P-values < 0.05 were considered statistically significant. The maps were produced using QGIS Geographic Information System (version 2.8.1) (48). Canton boundaries were obtained from the Swiss Federal Office of Topography (http://www.swisstopo.admin.ch).

Sequence accession numbers

All sequences from this study were submitted to GenBank under the accession numbers KU747015-KU747078, KP862870 and KP862872.

Results

Characteristics of the study population

A total of 75 FCV isolates collected during a previous nationwide FCV study (13) and originating from four healthy cats and 71 cats with suspected infection were included

in this study. Amplification of the PCR product and sequencing was successful for 66 FCV isolates from three healthy cats and 63 cats with suspected infection (Tables 1-3). The 66 cats were sampled in 22 veterinary practices located in 17 out of 26 cantons in Switzerland (Figure 1). The geographic distance (based on the postcodes) between the veterinary practices ranged from 4.7 km to 240 km (median = 92.4 km), and the distance between cat owners ranged from 0 km (same village) to 250 km (median = 95 km). Out of the 66 cats, 21 (32%) were pedigree cats; they belonged to seven different breeds (11 Maine Coons, 4 Norwegian Forest cats, 2 British Shorthair cats and 1 each of Oriental, Persian, Sacred Birman and Siamese cats). Fifty cats (78%) lived in multi-cat households (households with ≥ 2 cats), and 38 cats (59%) had outdoor access. Most of the cats were vaccinated against FCV (n = 46; 73%), and most had received at least a primary immunization (defined as two subsequent vaccinations within two to six weeks with the same vaccine strain, n = 33; 59%). Stomatitis, gingivitis or caudal stomatitis were the most common clinical signs (n = 48; 73%), and co-infections with other upper respiratory tract-associated pathogens were present in 44 (67%) of the cats.

FCV phylogeny and strain identification

Phylogenetic analysis included 66 nearly complete capsid (VP1) gene sequences (1700-1800 nt) from naturally infected cats, four FCV vaccine strains (FCV F9, FCV 255, G1 and 431) and 37 reference FCV sequences retrieved from GenBank. In general, the genetic heterogeneity among the capsid (VP1) gene sequences of the FCV isolates was very high. The resulting codon-based nucleotide alignment showed that individual nucleotides and their corresponding amino acid sites were either invariant or highly variable (data not shown). The estimated FCV phylogenies (Bayesian tree in Figure 2; ML tree in Supplementary Figure 1) were in accordance

with these sequence properties. Specifically, there was a notable lack of phylogenetic structure, and most of the internal nodes close to the root were poorly supported, with Bayesian posterior probabilities of < 0.8 and ML bootstrap scores of < 50%. A common property of these phylogenies was that their topologies exhibited short external branches and long internal branches. Further, the estimated Bayesian and ML topologies were incongruent. To quantify this, we placed the ML tree bootstrap scores onto the estimated Bayesian phylogeny (Figure 2 and Supplementary Figure 1), which illustrates the topological incongruences between the two phylogenies, especially at more ancestral nodes. Nodes near the tree tips tended to be better supported, with high Bayesian posterior probabilities closer to 1.0 and ML tree bootstrap scores > 50%.

Using a pairwise nucleotide genetic distance threshold of 20% in variable region E of the capsid (VP1) gene sequence, the isolates were assigned to 52 strains. Most of the strains (81%) were defined by only one isolate, with the exception of 10 strains (A-J) that contained two to three isolates (Figure 2). Within these multi-isolate strains (A-J), the pairwise genetic distances in variable region E of the FCV isolates ranged from 2% to 20%. Remarkably, both the Bayesian and the ML phylogenies showed two well-supported lineages above the strain level (denoted lineages 1 and 2 in Figure 2 and Supplementary Figure 1; Bayesian posterior probability = 1.0 and bootstrap scores > 80%). Lineage 1 comprised isolates from strains B and C and five single-strain FCV isolates. Lineage 2 comprised isolates from strains F-J plus fourteen single-strain FCV isolates. Both lineages contained not only FCV isolates from this study but also reference FCV isolates from a previous study from cats from Switzerland or Liechtenstein, which neighbours Switzerland (11). Outside these two lineages, the nodes that represent the common ancestor strains containing multiple

isolates (i.e., strains A, D and E) were well-supported, with Bayesian posterior probabilities = 1.0 and ML bootstrap scores equal to 100%. Conversely, within lineages 1 and 2, the nodes that represent the common ancestor of each multi-isolate strain were well-supported in some instances (C, I and J) but poorly supported in others (B, F, G and H), indicating a difference between the genetic and patristic distances. The internal nodes within strains A, C, F and H tended to be supported with Bayesian posterior probabilities > 0.8 but not with bootstrap scores > 80%, whereas most of the nodes between the strains were poorly supported (Bayesian posterior probability < 0.8 and bootstrap scores < 50%).

The geographic origin of the FCV isolates assigned to different strains and lineages is indicated in Figures 3a and 3b, respectively. The maximal geographic distance between FCV isolates within a given strain (based on the postcodes of the cat owners) ranged from 0 km (strain J) to 209 km (strain F). Both isolates of strain J (isolates 32 and 145) were derived from one village, and all isolates of strains A (isolates 49, 83 and 106) and D (isolates 68 and 155) were from the same canton in Switzerland.

All reference sequences retrieved from GenBank that were included in the phylogenetic analyses were placed outside strains A-J, except for the FCV vaccine strain F9, which was located in strain D together with two isolates of this study (isolates 68 and 155). These two isolates were obtained from the same veterinary practice and from two cats that had received a FCV vaccination (with Feligen® CRP, Virbac; containing FCV F9 vaccine strain) 41 days and 70 days prior to sample collection.

Association of FCV phylogeny with different traits

All traits listed in Tables 1-3 and the "veterinary practice" trait were tested for association with FCV phylogeny. For each trait, we tested whether we could reject the null hypothesis that the trait is randomly distributed with respect to the tree topology.

A strong statistical association was found between the FCV phylogeny and the “pedigree” trait (Table 1; $p < 0.01$ using the AI and PS statistics). Ancestral character state estimation of this trait showed that most of the FCV isolates (17 out of 21) from pedigree cats belonged to lineage 2 and formed a monophyletic clade dominated by pedigree cats (Figure 4). Within lineage 2, 10 of the 17 FCV isolates from pedigree cats were derived from Maine Coon cats, which was the predominant breed in our study population. Within lineage 2, eight isolates from pedigree cats belonged to strains F, G, H and I, and nine isolates represented single-isolate strains. The remaining four isolates from pedigree cats were single-isolate strains located outside of lineages 1 and 2. The association of FCV phylogeny with "pedigree cat" could not be explained by a geographical clustering of the pedigree cats because the cats originated from 11 different cantons, and the samples were collected in 14 different veterinary practices (Figure 1). Furthermore, when the genetic distance of pedigree and non-pedigree isolates was separately compared to geographic distances between owners or veterinary practices, no correlation was found (Supplementary Figure 2). We further assessed whether the pedigree cats in this study exhibited some characteristics that were different from the non-pedigree cats that could potentially account for the observed association of the “pedigree” trait with FCV phylogeny (Supplementary Table 1). The analysis revealed that the pedigree cats were more commonly kept by cat breeders ($p_{\chi 2} < 0.01$), lived more commonly in

multi-cat households ($p_{\chi 2} = 0.02$) and without outdoor access ($p_{\chi 2} < 0.01$), and were less commonly treated with immunosuppressive ($p_{\chi 2} = 0.02$) and antibiotic medications ($p_{\chi 2} = 0.04$) than non-pedigree cats. The pedigree cats had more commonly received a primary immunization against FCV ($p_{\chi 2} = 0.03$), and the FCV vaccine strains used for immunization were different from those used for the non-pedigree cats ($p_{\chi 2} < 0.01$). Furthermore, pedigree cats more commonly suffered from lingual and oral ulcerations ($p_{\chi 2} < 0.01$; data not shown) than the non-pedigree cats.

There was also a significant association of the “veterinary practice” trait with FCV phylogeny ($p < 0.01$ using AI statistic, $p = 0.05$ using PS statistics). Ancestral state reconstruction indicated that some multi-isolate strains (strains A, D and J) were localised to individual veterinary practices; for strain C, two out of the three isolates came from the same practice (Figure 4). The time span between collections of closely related isolates within one veterinary practice ranged from six days (strain C; isolates 65 and 81) to seven weeks (strain J; isolates 32 and 145). The FCV isolates within the remaining six multi-isolate strains derived from different Swiss cantons and were collected in different veterinary practices. Overall, the strain diversity collected in each veterinary practice was high: in 19 out of 22 veterinary practices, the number of collected samples was equal to the number of FCV strains.

Looking at the different forms of husbandry type (private, cat breeder and others), the association with FCV phylogeny was inconclusive ($p = 0.02$ using the AI statistic; $p = 0.2$ using the PS statistic; Table 1). A questionable statistical association with FCV phylogeny was also found for the "primary FCV immunization" trait ($p = 0.06$ using the AI statistic, $p = 0.02$ using the PS statistic, Table 2). Finally, there was an inconclusive association of FCV phylogeny with the vaccine strain used for immunization ($p = 0.04$ using the AI statistic, $p = 0.08$ using the PS statistic, Table 2).

Because "pedigree" was significantly associated with husbandry type, primary immunization of the cats and the vaccine strain used for immunization (Supplementary Table 1), these associations could have acted as confounding variables in the single-trait analyses. All other variables investigated in this study showed no significant association with FCV phylogeny (Tables 1 - 3).

Correlation between pairwise genetic and geographical distances

Given the weak statistical support for many phylogenetic nodes, we further explored the spatial clustering of FCV isolates by plotting pairwise genetic distances against pairwise geographical distances (Figure 5). Pairwise geographical distances were calculated between the locations of the cat owners and also between the locations of the veterinary practices where the samples had been collected. We observed weak associations between genetic and spatial distances in both cases (Pearson correlation $r = 0.036$, $p = 0.245$ for distances between cat owners; Pearson correlation $r = 0.033$, $p = 0.268$ for distances between veterinary practices).

Discussion

Investigating the genetic diversity and phylogeny of FCV isolates is a prerequisite to understanding the epidemiology and pathogenesis of FCV and may assist in the development of efficacious vaccine strategies. This study provides, for the first time, a nationwide analysis of FCV isolates based on the nearly complete capsid (VP1) gene. We identified 52 different strains circulating in the Swiss cat population and two well-supported lineages above the strain level that contained 24 and 9 isolates. The remarkably high strain diversity observed in the present study is in agreement with three recent studies that addressed FCV phylogeny within communities (49), at the country level in the UK (27) and in different European countries (28). These three

studies were all based on partial capsid (VP1) gene sequences (i.e., variable regions C and E of the ORF2 of FCV) (27, 28, 49) and were hampered by a lack of phylogenetic signal above the strain level caused by sequence saturation. To overcome this limitation, in this study, we analysed the almost complete capsid (VP1) gene of the FCV isolates, which included more conserved domains of the capsid protein. The resolution of the phylogeny in our study was thus greater than in other studies (27, 28). Nonetheless, there was still a lack of deep phylogenetic structure; most internal nodes close to the phylogeny root were poorly supported, reflecting the exceptionally polymorphic nature of variable sites within the FCV capsid (VP1) gene.

In agreement with previous studies, we found remarkably high strain diversity among FCV isolates in Switzerland. Strains containing multiple isolates comprised no more than three different isolates, which resulted in a maximum prevalence of 4.5% for any given FCV strain of this study. This remarkably high strain diversity is also influenced by the strain definition used. In accordance with previous studies, a strain was defined by > 20% genetic distance in variable region E of the capsid (VP1) gene and included 235 bp of variable region E. This definition is based on studies that showed that related FCV isolates in endemically infected colonies show up to 16% genetic distance, whereas unrelated isolates show > 20% distance in variable region E of VP1 (23, 24). The FCV strain definition therefore applies to closely related isolates that are epidemiologically and spatially linked (23, 24). However, it is sometimes used ambiguously, as some studies applied the genetic distance definition to 235 bp of the variable region E (23, 25), whereas others applied it 420 bp (20) to 529 bp amplicons (28, 50) of variable regions C and E. These differences are important given the very high variation in genetic diversity among sequence regions within the capsid (VP1) gene. Our analyses also identified two well-supported lineages above

the strain level, lineages 1 and 2, that included 10 and 26 FCV isolates, respectively. The isolates within lineages 1 and 2 showed 15 - 33% and 12 - 34% pairwise genetic distances, respectively, in variable region E of the capsid (VP1) gene. Sequences with genetic distances >20% that cluster together with bootstrap scores >80% have so far only been documented in one study of cats in the UK, but was a rare finding (27).

We identified a strong statistical association between FCV phylogeny and the “pedigree” trait. Ancestral character state estimation revealed that 17 out of 21 FCV isolates from pedigree cats belonged to lineage 2 and formed a monophyletic and well-supported clade mostly comprising pedigree cats, indicating an epidemiological link between these isolates. It could be hypothesized that pedigree cats represent a new environmental niche to which FCV might become adapted. To the best of our knowledge, this is the first report where FCV genetic diversity has been successfully correlated with characteristics of the host. The association with pedigree might be a caused by a common route of transmission of FCV strains among pedigree cats in Switzerland, e.g., during cat exhibitions or within breeding catteries. Alternatively, genetic characteristics of pedigree cats might explain the strong association between FCV phylogeny and the “pedigree” trait. Most pedigree cats in this study were Maine Coon cats, and 10 out of 11 FCV isolates from Maine Coon cats were located in lineage 2. However, FCV isolates from other cat breeds in this study were also predominantly placed in lineage 2. Maine Coon cats might be overrepresented in the present study because the cat population of this study was not representative of the Swiss cat population.

In agreement with a previous study (27), we found only weak associations between the genetic and spatial distances of FCV isolates. This was the case for both the

location of the cat owners and the location of the veterinary practices where the samples had been collected. Recent studies reported that FCV strains are confined to close geographic regions or single countries (27, 28). A study in cats in the UK found only two FCV strains detected >100 km apart, and the most widespread strain contained only variants of FCV F9, a common FCV vaccine strain. The authors of that study concluded that FCV seems to have only limited ability for widespread geographic spread and that the movement of persistently infected cats is the most likely reason for dissemination (27). In our study, all but one multi-isolate strain (exception strain D) and both lineages 1 and 2, contained only isolates from cats from Switzerland and Liechtenstein, a neighbouring country of Switzerland. However, the maximal geographical distance between FCV isolates of multi-isolate strains was 209 km (strain F), and geographic distances of >100 km within a multi-isolate strain were not uncommon. We only identified two FCV F9 variants (isolates 68 and 155, strain D), and in contrast to the UK study, they were not geographically dispersed and originated from two cats from the same veterinary practice. There could be several reasons for the differences between our results and those of the UK study. First, the shorter distances between sampling sites in our study (median 92.4 km) compared to the UK study (over 300 km for the majority of practices (27)) allowed us to also explore intermediate distances of FCV dissemination. Second, our study included a higher percentage (0.004%) of the total national cat population (estimated 1.5 million cats, http://www.tierschutz.com/publikationen/heimtiere/infothek/katzen_hunde/mb_katzen_kastration.pdf) than the UK study (0.002%, estimated 8 million cats, http://www.pfma.org.uk/pet-population-2008-2012), which might have allowed us to detect less prevalent FCV strains. Third, only one FCV isolate per household was included in our study, whereas isolates collected from cats from the same household were included in the UK study, which could have altered the geographical

footprint of FCV. Fourth, our study excluded cats with recent FCV vaccination to avoid the inclusion of FCV vaccine strains that can occasionally be detected up to three weeks after vaccination (51-53). The most geographically widespread FCV strains in our study (strains F to I) were predominantly obtained from pedigree cats. Pedigree cats might be moved over longer geographic distances, i.e., for breeding or for cat exhibitions. The inclusion of a relatively large number of pedigree cats in our study could therefore also account for the observed wide geographic dispersal of some FCV strains.

In agreement with a previous study, the overall strain diversity collected in veterinary practices was remarkably high (27). In 19 out of 22 practices, the number of collected samples was equal to the number of FCV strains. Nevertheless, we found a significant association between the “veterinary practice” trait and the FCV phylogeny. This association could be because several multi-isolate strains (A, C, D and J) were almost exclusively collected in single veterinary practices. The existence of closely related FCV isolates in one veterinary practice has also been previously reported (27). Indirect transmission of FCV is common, since this non-enveloped RNA virus is highly resistant to many disinfectants and can remain infectious for up to one month in the environment (54). If hygiene measures are insufficient, indirect transmission could play a role in the environment of a veterinary practice. However, this would require that the cats were presented and infected in the veterinary practices prior to sample collection. Alternatively, contamination of swabs with FCV from the practice environment could have caused PCR-positive results. However, all except one of the cats from which the isolates of strains A, C, D and J were obtained showed clinical signs consistent with FCV infection. Furthermore, all sample collection material was provided to the veterinary practices together with detailed instructions on how to

properly collect the samples. Finally, the detection of closely related FCV isolates in one veterinary practice could reflect the transmission of this FCV strain between cats in the catchment area of that practice. The cats infected with variants of the multi-isolate strains A, D or J lived in rather close proximity to each other (within 16 km apart), although this was not the case for the cats infected with variants of strain C (up to 62 km apart). Furthermore, only five out of nine cats in strains A, C, D and J were allowed outdoors. However, FCV can also be indirectly transmitted between cats via fomites, which could have accounted for the infections of the indoor cats.

Only two isolates closely related to the FCV vaccine strain F9 were detected in this study (isolates 68 and 155, strain D); the isolates showed <3% genetic divergence from the vaccine strain in the variable region E. Both samples were collected in a single veterinary practice and derived from two cats that had been vaccinated with a vaccine containing FCV F9 41 and 70 days prior to sample collection, respectively. FCV F9 is contained in several modified-live virus vaccines licensed in Switzerland, and a short-term oral shedding of FCV F9 for some days up to three weeks after vaccination has been reported (51-53). Previous studies have also occasionally reported the isolation of FCV F9 variants from the general cat population (27, 50, 55), but in contrast to the present study, recent vaccination was not an exclusion criterion in these studies. A very recent multinational study of cats from different European countries did not detect any isolates related to FCV F9; cats vaccinated within one month prior to sample collection were excluded from that study (28). The detection of FCV F9 variants in the present study up to 70 days after vaccination could be explained by an inadvertent infection of the cats with the FCV vaccine strain, as reported after the licking of accidently spilled vaccine material from the fur of the cats. In such cases, clinical signs of FCV infection and prolonged shedding of the vaccine

virus (several weeks to a few months) can occur (51). Alternatively, the two cats might have been exposed to an FCV F9-like field variant, but this seems less likely because cat 155 was kept strictly indoors as a single cat and lived 16 km away from cat 68. Finally, prolonged shedding of FCV F9 after the correct application of the vaccine might occur in cats with a severe immune deficiency. While cat 68 was a healthy young cat presented for castration, cat 155 presented with fever, apathy, ocular discharge and gingivitis and tested PCR-positive for feline immunodeficiency virus (FIV) in RT-qPCR (13). However, it seems improbable that immunosuppression due to FIV infection explains the extended time of shedding of FCV after vaccination in cat 155. Prolonged FCV shedding was not observed in FIV-positive cats in an experimental study (56), and severe immunodeficiency usually occurs only during long-term FIV infection (57); cat 155 was only five months old at the time of sampling.

Conclusion

Our study indicates that FCV lineages above the strain level can sometimes be identified if genetic data from more conserved regions of the FCV genome are included in phylogenetic analyses. FCV phylogeny was significantly associated with the pedigree status of the sampled animals, and the isolates from most pedigree cats were placed in lineage 2 in this study. All but one strains and both lineages identified in this study were restricted to cats in Switzerland or Liechtenstein. Within Switzerland, we observed a greater geographic dispersal of FCV strains than previously reported. Variants of the FCV F9 vaccine strain were very rarely detected and may be assigned to the inadvertent infection of cats by oral intake of spilled vaccine material. The resolution of the FCV phylogeny in this study was greater than that in previous studies, which can be attributed to the use of more conserved regions of the capsid (VP1) gene. Nonetheless, phylogenetic analyses were still

hampered by sequence saturation. To overcome this limitation and to further resolve the phylogenetic relationships of FCV isolates, future studies should extend genetic analyses to longer sequences of FCV.

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Authors' contribution: BW and RHL conceived and conducted the study. BW supervised the study. AMS and AB conducted the laboratory experiments. MLM was responsible for all laboratory aspects. JT, OGP, VC and AS performed the genetic and phylogenetic analyses. AMS and JT drafted the manuscript. RHL and BW edited the manuscript. All authors read and approved the final version of the manuscript.

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Figure legends

Figure 1: Map of Switzerland and its cantons, showing the origin of the FCV isolates sequenced in this study. Coordinates were calculated using the home address of the cat owners, and the map was produced using QGIS (48).

Figure 2: Bayesian phylogenetic tree estimated from 109 FCV capsid (VP1) gene nucleotide sequences, including reference isolates and isolates from this study. Statistical support for phylogenetic nodes corresponds to the Bayesian posterior probabilities and ML bootstrap scores (100 replicates, see Supplementary Figure 1), separated by the slash character. Hyphens replace posterior probabilities <0.8 and bootstrap scores <50%. Tip labels are coloured by sampling locations: Swiss isolates from this study are in red, European reference isolates in green, North American reference isolates in blue, Oceanian reference isolates in purple, and Asian reference isolates in brown. Grey boxed areas denote multi-isolate strains A to J (for details see text). Minimum-maximum genetic diversity (%) and minimum-maximum geographical distances (km) within each multiple-isolate strain are indicated next to the corresponding strain name. Circled numbers indicate denoted lineages 1 and 2.

Figure 3: Maps of Switzerland and its cantons displaying (a) the distribution of the multi- and single-isolate strains and (b) the distribution of the FCV isolates assigned to lineage 1, 2 or no lineage. Both isolates of strain J originated from the same village and are indicated as a single dot in Figure 3a. Coordinates were calculated using the home address of the cat owners, and the map was produced using QGIS (48).

Figure 4: Bayesian FCV phylogenetic tree upon which ancestral trait state reconstructions have been superimposed. Tip circles in red and in blue show FCVs isolated from non-pedigree cats and pedigree cats, respectively. Node circles

indicate the parsimonious ancestral trait state reconstruction of the “pedigree” trait. Dashed branches represent lineages for which ancestral character state reconstruction of the “veterinary practice” trait was non-ambiguous. The colours of the dashed branches correspond to the veterinary practices.

Figure 5: Comparison of pairwise genetic distances (%) and pairwise geographical distances obtained from the isolates in this study using variable region E of the capsid (VP1) gene. Panel A shows the correlation obtained if the pairwise geographic distances are calculated between the locations of the cat owners, and panel B shows the correlation obtained if the pairwise geographic distances are calculated between the locations of the veterinary practices.

Figures

Figure 1:

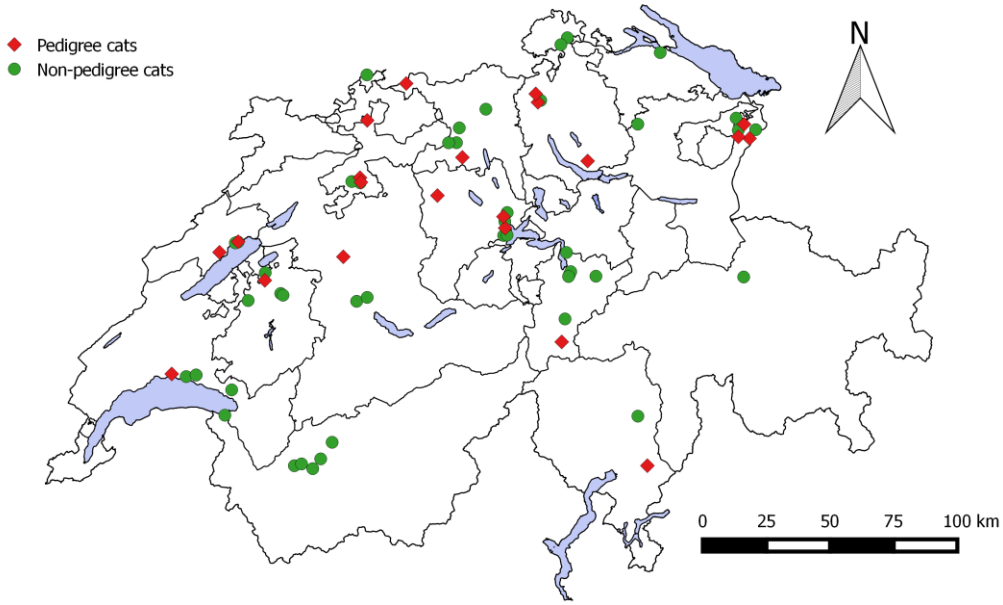


Figure 2:

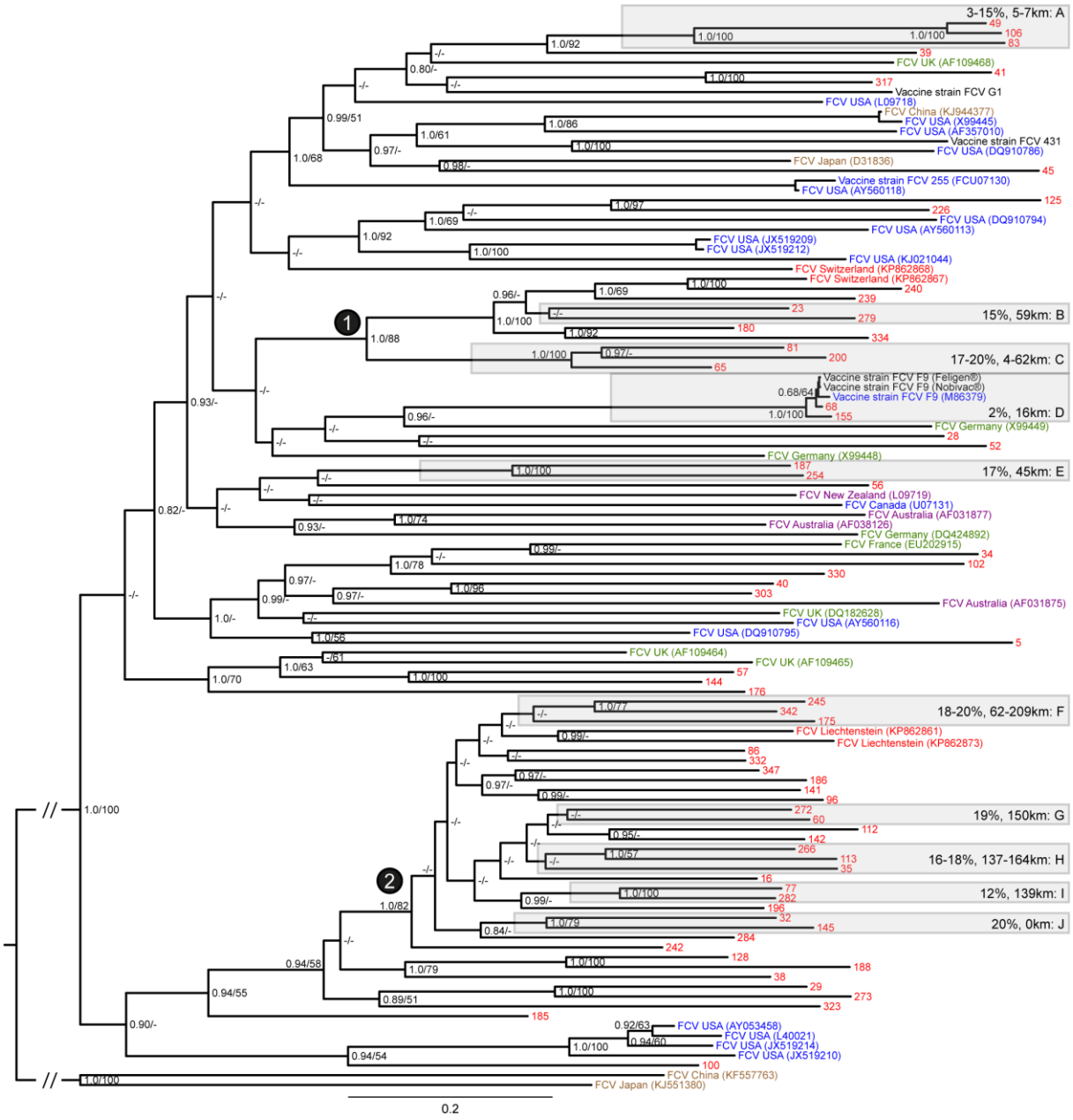

3-15%, 5-7km: A
15%, 59km: B
17-20%, 4-62km: C
2%, 16km: D
17%, 45km: E
18-20%, 62-209km: F
19%, 150km: G
16-18%, 137-164km: H
12%, 139km: I
20%, 0km: J
FCV UK (AF109468)
Vaccine strain FCV G1
FCV USA (L09718)
FCV China (KJ944377)
FCV USA (X99445)
FCV USA (AF357010)
Vaccine strain FCV 431
FCV USA (DQ910786)
FCV Japan (D31836)
Vaccine strain FCV 255 (FCU07130)
FCV USA (AY560118)
FCV USA (DQ910794)
FCV USA (AY560113)
FCV USA (JX519209)
FCV USA (JX519212)
FCV USA (KJ021044)
FCV Switzerland (KP862868)
FCV Switzerland (KP862867)
Vaccine strain FCV F9 (Feligen®)
Vaccine strain FCV F9 (Nobivac®)
Vaccine strain FCV F9 (M86379)
FCV Germany (X99449)
FCV Germany (X99448)
FCV New Zealand (L09719)
FCV Canada (U07131)
FCV Australia (AF031877)
FCV Australia (AF038126)
FCV Germany (DQ424892)
FCV France (EU202915)
FCV Australia (AF031875)
FCV UK (DQ182628)
FCV USA (AY560116)
FCV USA (DQ910795)
FCV UK (AF109464)
FCV UK (AF109465)
FCV Liechtenstein (KP862861)
FCV Liechtenstein (KP862873)
FCV USA (AY053458)
FCV USA (L40021)
FCV USA (JX519214)
FCV USA (JX519210)
FCV China (KF557763)
FCV Japan (KJ551380)
0.2

Figure 3A:

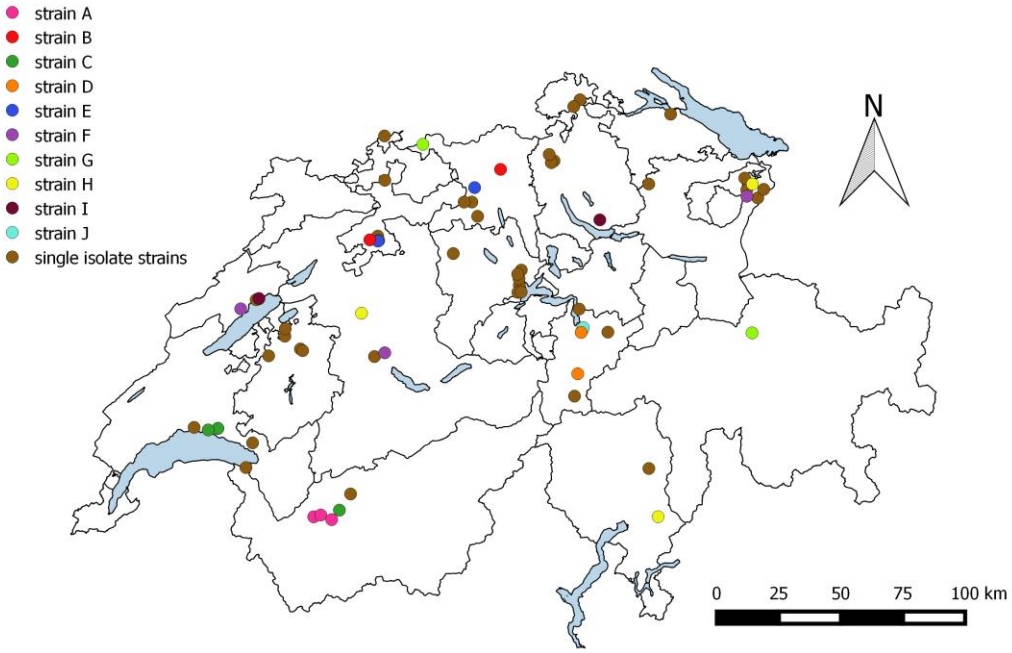


Figure 3B:

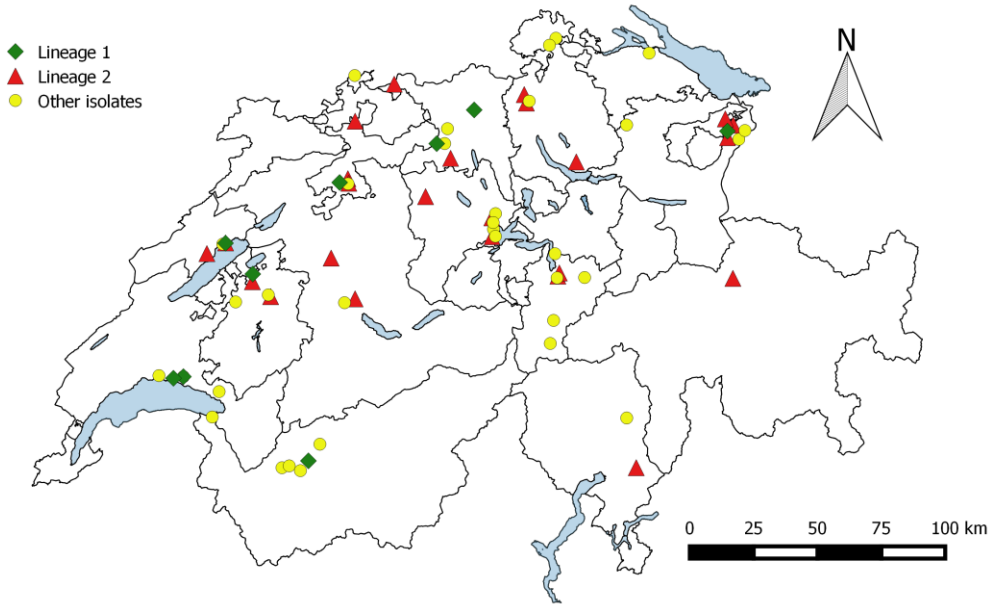


Figure 4:

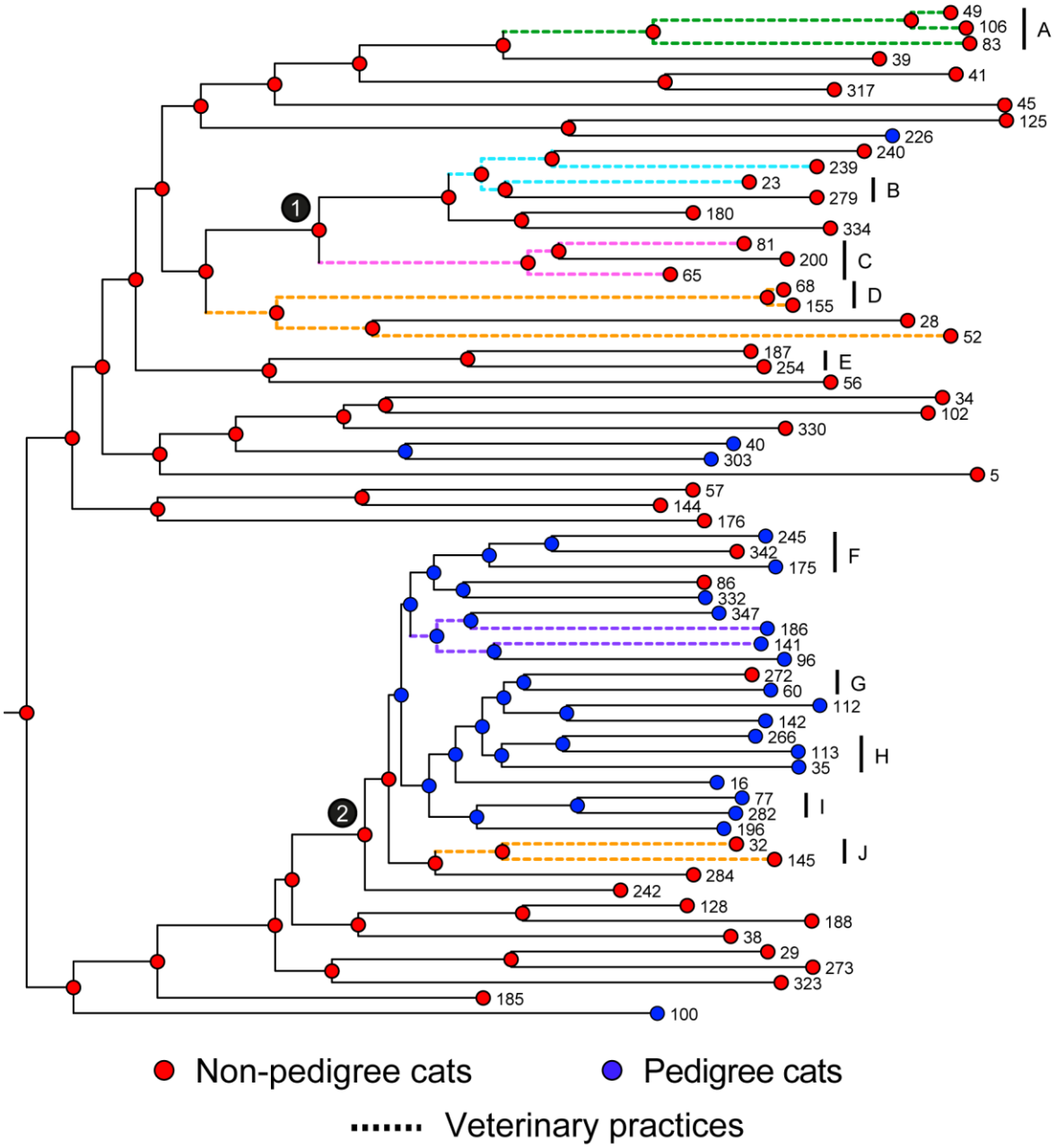
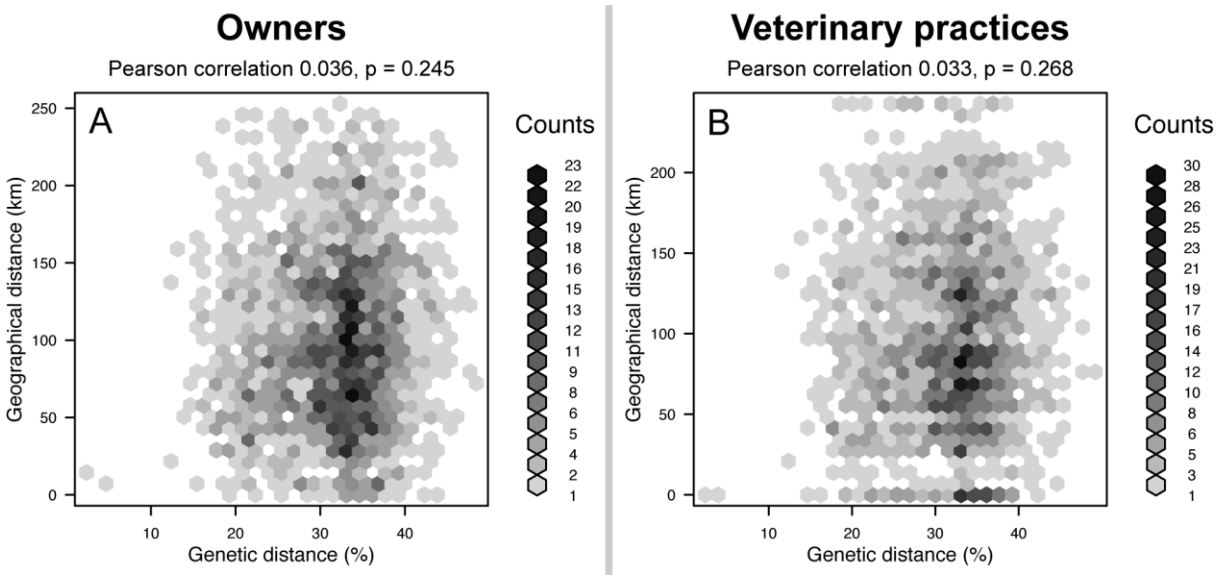

49
106
83
A
39
41
317
45
125
226
240
239
23
279
B
180
334
81
200
C
65
68
155
D
28
52
187
254
E
56
34
102
330
40
303
5
57
144
176
245
342
175
F
86
332
347
186
141
96
272
60
G
112
142
266
113
35
H
16
77
282
196
I
32
145
J
284
242
128
188
38
29
273
323
185
100
1
2
Non-pedigree cats
Pedigree cats
Veterinary practices

Figure 5:


Owners
Pearson correlation 0.036, p = 0.245
A
Geographical distance (km)
Genetic distance (%)
Counts
Veterinary practices
Pearson correlation 0.033, p = 0.268
B
Geographical distance (km)
Genetic distance (%)
Counts

Tables

Table 1: Characteristics of the 66 FCV-positive cats and association with FCV phylogeny. *P values < 0.05 are shown in bold.*

Parameter	Median	Lower 95% CI[#]	Upper 95% CI[#]	AI[&] (P value)	PS[§] (P value)
Age (years)	3.8	0.25	13.7		
Parameter	**Proportion**	**Lower 95% CI[#]**	**Upper 95% CI[#]**	**AI[&] (P value)**	**PS[§] (P value)**
Sex (male)	0.62	0.49	0.74	0.13	0.26
Intact reproductive status*	0.46	0.33	0.59	0.62	0.81
Pedigree	0.32	0.21	0.44	**< 0.01**	**< 0.01**
Breed				**< 0.01**	**< 0.01**
Maine Coon	0.17	0.09	0.28		
British Shorthair	0.03	0.00	0.11		
Norwegian Forest Cat	0.06	0.02	0.15		
Other breeds	0.06	0.02	0.15		
Multi-cat household*	0.78	0.66	0.88	0.56	0.24
Group size*				0.22	0.23
with 2-3 cats/group	0.40	0.28	0.53		
with 4-5 cats/group	0.16	0.08	0.27		
>5 cats/group	0.22	0.13	0.35		
Husbandry type				**0.02**	0.2
Private	0.79	0.67	0.88		
Cat breeder	0.09	0.03	0.19		
Other husbandry	0.12	0.05	0.23		
Outdoor access*	0.59	0.46	0.71	0.56	0.12
Immunosuppressive therapy	0.15	0.08	0.26	0.43	0.31
Antibiotic therapy	0.32	0.21	0.44	0.16	0.23
Antiviral therapy*	0.03	0.00	0.11	0.25	1.0

[#]CI, confidence interval, [&]AI, association index, [§] PS, parsimony score, *data missing for single cats (reproductive status, n = 5; multi-cat household, n = 2; group size, n = 3; outdoor access, n = 1, antiviral therapy, n = 1)

Table 2: FCV vaccination status of the 66 FCV-positive cats and association with FCV phylogeny. *P values < 0.05 are shown in bold.*

Parameter	Proportion	Lower 95% CI[#]	Upper 95% CI[#]	AI[&] (P value)	PS[§] (P value)
FCV-vaccinated*	0.73	0.43	0.68	0.38	0.25
FCV vaccine strain*				**0.04**	0.08
F9	0.32	0.21	0.45		
Purevax	0.06	0.02	0.16		
Other vaccine strain	0.35	0.23	0.48		
Primary immunization[‡]	0.59	0.45	0.72	0.06	**0.02**

[#]CI, confidence interval, [&]AI, association index, [§] PS, parsimony score, *data missing for single cats (FCV vaccination status, n = 3; FCV vaccine strain, n = 3; primary immunization, n = 10), [‡]primary immunization was defined as two subsequent vaccinations within 2 to 6 weeks with the same FCV vaccine strain

Table 3: Health status, clinical signs and co-infections with other upper respiratory tract-associated pathogens in the 66 FCV-positive cats and association with FCV phylogeny.

Parameter	**Proportion**	**Lower 95% CI[#]**	**Upper 95% CI[#]**	**AI[&] (P value)**	**PS[§] (P value)**
Healthy	0.05	0.01	0.13	0.31	1.0
Sneezing and/or nasal discharge*	0.32	0.21	0.45	0.61	0.93
Conjunctivitis and/or ocular discharge	0.39	0.28	0.52	0.44	0.31
Salivation	0.38	0.26	0.51	0.79	0.87
Lingual and/or oral ulcerations	0.30	0.20	0.43	0.79	0.31
Stomatitis and/or gingivitis and/or caudal stomatitis	0.73	0.60	0.83	0.25	0.52
Cutaneous ulcerations and/or cutaneous oedema	0.05	0.01	0.13	0.07	1.0
Limping and/or swollen joints	0.06	0.02	0.15	0.25	1.0
M. felis PCR-positive	0.56	0.43	0.69	0.06	0.06
FHV-1 PCR-positive	0.23	0.13	0.35	0.25	0.62
C. felis PCR-positive	0.05	0.01	0.13	0.25	1.0
B. bronchiseptica PCR-positive	0.05	0.01	0.13	0.43	1.0

[#]CI, confidence interval, [&]AI, association index, [§]PS, parsimony score, *data from one cat missing

Appendix

Supplementary Table

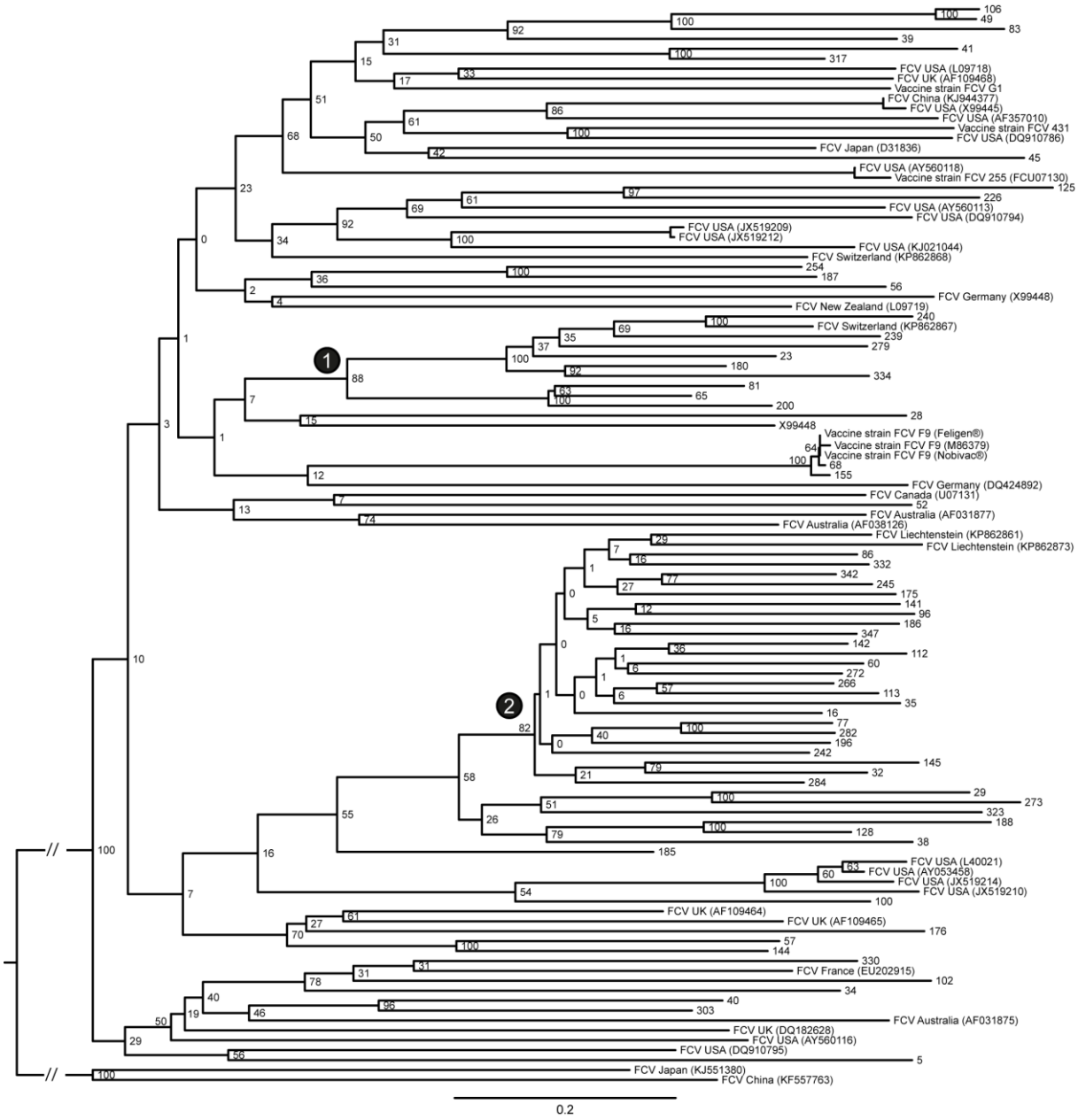
Comparison of characteristics between the 45 non-pedigree and the 21 pedigree cats. P values < 0.05 are shown in bold.

Parameter	Non-pedigree cats (n = 45)			Pedigree cats (n = 21)			
	Median	**Lower 95% CI[#]**	**Upper 95% CI[#]**	**Median**	**Lower 95% CI[#]**	**Upper 95% CI[#]**	**P value**
Age (years)	5.0	0.35	15.40	1.75	0.18	9.08	0.10
Parameter	**Proportion**	**Lower 95% CI[#]**	**Upper 95% CI[#]**	**Proportion**	**Lower 95% CI[#]**	**Upper 95% CI[#]**	**P value**
Sex (male)	0.67	0.51	0.80	0.52	0.30	0.74	0.27
Intact reproductive* status	0.45	0.30	0.61	0.47	0.24	0.71	0.88
Multi-cat household*	0.70	0.54	0.83	0.95	0.76	1.00	**0.02**
Group housing*							0.16
2-3 cats/group	0.43	0.28	0.59	0.33	0.15	0.57	
4-5 cats/group	0.10	0.03	0.23	0.29	0.11	0.52	
>5 cats	0.17	0.07	0.31	0.33	0.15	0.57	
Husbandry type							**<0.01**
Private	0.82	0.68	0.92	0.71	0.48	0.89	
Cat breeder	0.00	0.00	0.00	0.29	0.11	0.52	
Other husbandry	0.18	0.08	0.32	0.00	0.00	0.00	
Outdoor access*	0.75	0.60	0.87	0.24	0.08	0.47	**<0.01**
Immunosuppressive therapy	0.22	0.11	0.37	0.00	0.00	0.00	**0.02**
Antibiotic therapy	0.40	0.26	0.56	0.14	0.03	0.36	**0.04**
Antiviral therapy*	0.02	0.00	0.12	0.05	0.00	0.25	0.55
FCV-vaccinated*	0.67	0.52	0.81	0.85	0.62	0.97	0.14
FCV vaccine strain*							**0.01**
F9	0.21	0.10	0.36	0.55	0.32	0.77	
Purevax	0.02	0.00	0.12	0.15	0.03	0.38	
Others	0.44	0.29	0.60	0.15	0.03	0.38	
Primary immunization*,‡	0.49	0.32	0.66	0.79	0.54	0.94	**0.03**

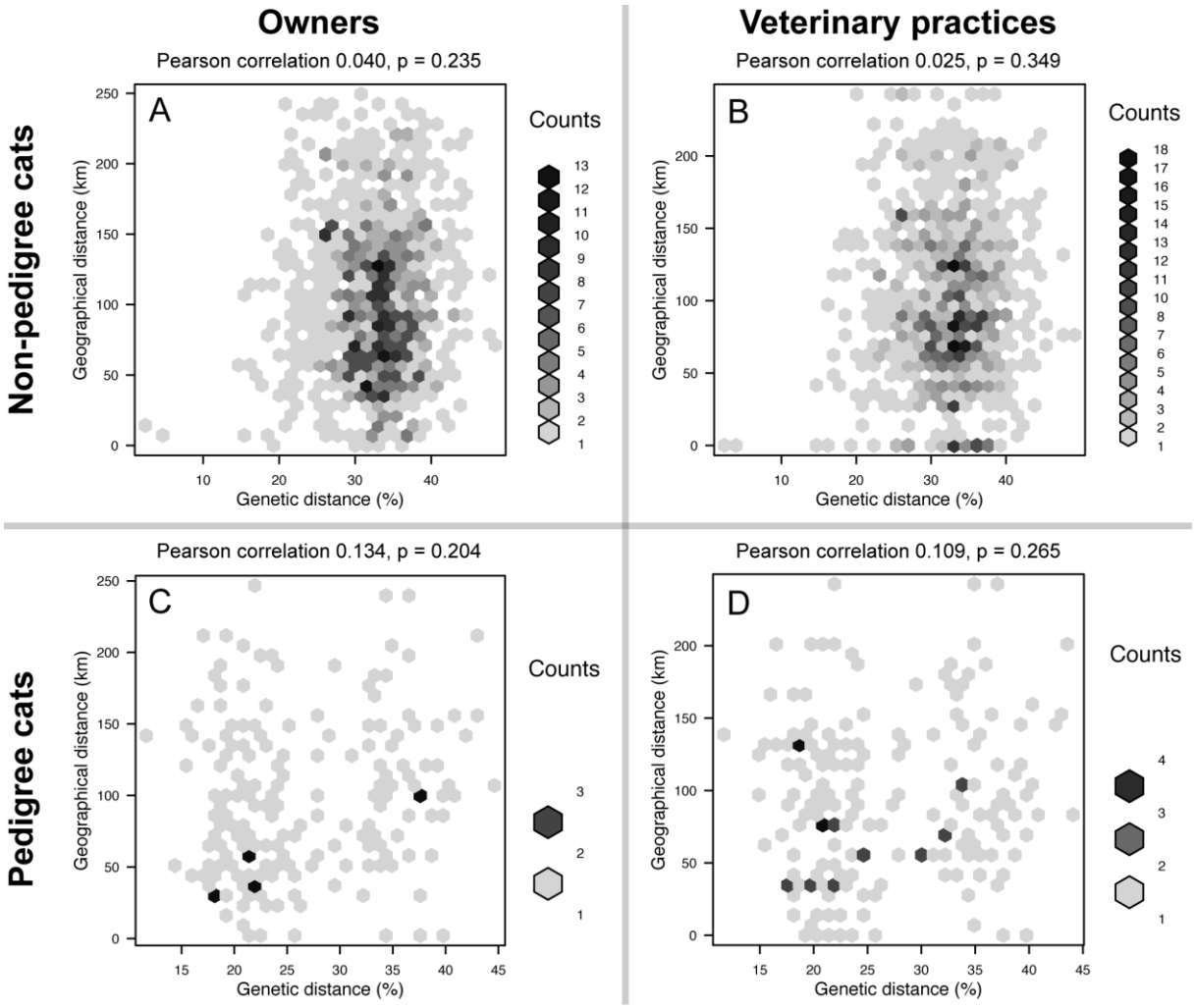
[#]CI, confidence interval, * data missing for single cats (reproductive status, n = 5; multi-cat household, n = 2; group housing, n = 3; outdoor access, n = 1; antiviral therapy, n = 1; FCV vaccination, n = 3; FCV vaccine strain, n = 3; primary immunization, n = 10), ‡primary immunization was defined as two subsequent vaccinations within 2 to 6 weeks with the same FCV vaccine strain

Supplementary Figures

Supplementary Figure 1: Maximum likelihood phylogenetic tree estimated from 109 FCV capsid gene nucleotide sequences, including reference isolates and isolates from this study. Statistical support for the phylogenetic nodes was assessed using a bootstrap approach (100 replicates).



Supplementary Figure 2: Comparison of pairwise genetic distances (%) and pairwise geographical distances obtained from the isolates in this study based on variable region E of VP1. Panels A and C show the correlations obtained if the pairwise geographic distances were calculated between the locations of the cat owners and included only samples isolated from non-pedigree cats. Panels B and D show the correlations obtained if the pairwise geographic distances were calculated between the locations of the veterinary practices and included only samples isolated from pedigree cats.



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Curriculum Vitae

Andrea Monika Spiri

Geburtsdatum: 11.02.1989

Geburtsort: Frauenfeld

Nationalität: Schweizerin

Heimatort: Amlikon-Bissegg

Schulausbildung

Aug. 1995 – Jul. 2001	Primarschule in Märstetten, Schweiz
Aug. 2001 – Jul. 2003	Oberstufe in Märstetten, Schweiz
Aug. 2003 – Jul. 2007	Kantonsschule in Frauenfeld, Schweiz
29. Jun. 2007	Maturitätsausweis, Kantonsschule Frauenfeld, Schweiz

Studium

Sept. 2007 – Jan. 2013	Studium Veterinärmedizin, Vetsuisse-Fakultät, Universität Zürich, Schweiz
30. Sept. 2012	Masterabschluss „Master of Veterinary Medicine” Universität Zürich, Schweiz
23. Jan. 2013	Eidgenössische Prüfung Veterinärmedizin (Staatsexamen), Universität Zürich, Schweiz
Mär. 2013 – Mai 2016	Anfertigung der Dissertation unter der Leitung von Prof. Regina Hofmann-Lehmann am Departement für Nutztiere, Veterinärmedizinisches Labor, der Vetsuisse-Fakultät, Universität Zürich, Schweiz